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(54) Title: TNF–R2 COMPLEX POLYGENIC DISEASE GENE MARKER

(57) Abstract

A method of testing an individual predisposed to or who may be at possible risk of developing a complex polygenic disease including but not restricted to coronary artery disease, essential hypertension, hyperlipidemia, non–insulin dependent diabetes mellitus, or diabetic neuropathy to ascertain whether there is an elevation in their risk of acquiring one or more of these conditions, the method comprising determination of the genotype of the individual for a polymorphism in the tumor necrosis factor receptor superfamily member 1B gene that encodes tumor necrosis factor receptor 2, wherein the presence of one allele of the polymorphism is indicative of an increased risk over an individual with a contrasting allele of the polymorphism.

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TNF-R2 complex polygenic disease gene marker

Technical Field

The present invention relates to a method of identifying a gene or a linked gene region responsible for contributing to the causation of a complex polygenic disease in a patient and for predicting the general susceptibility of an individual predisposed to or having a complex polygenic disease such as coronary artery disease, hypertension, hyperlipidemia or non-insulin-dependent diabetes mellitus or their complications.

Background

Cardiovascular disease is a major problem in developed nations, being responsible for close to 50% of total mortality. Such common conditions include coronary artery disease (CAD), high blood pressure (essential hypertension), atherosclerosis, hyperlipidemia, obesity and non-insulin-dependent diabetes mellitus (NIDDM). Individuals with one of these conditions are more likely to have one or more of the others as well. Terms such as "Syndrome X" or "the metabolic syndrome" have therefore been used to describe this collection of afflictions. All increase risk of heart attack (myocardial infarction), heart failure, stroke, and renal failure. The cause of such polygenic conditions is a combination of genetic background and environmental factors such as a diet high in saturated fat, and/or sodium chloride, lack of exercise, smoking, alcohol, and other factors. The traits are quantitative (i.e, severity varies over a measurable range) and complex (i.e., multiple genes are involved).

Determination of the etiology of these conditions is thus a major international research endeavour. In the case of hypertension, over the past several years several rare forms of early onset having characteristic features have been solved, revealing a monogenetic defect in individual cases. But these differ from essential hypertension and the other conditions, which all tend to be of late onset and are polygenic. The latter makes identification of the genes responsible very much more difficult.

Two basic approaches are used to identify polymorphisms that are either the cause of genetic disease or are markers for a causative variant nearby, viz. association (case-control) studies and linkage studies. Association studies involve testing of a patient population of unrelated affected individuals with a control unaffected population, matched as far as is possible for genetic background. Linkage studies look for co-inheritance of alleles of genetic markers and so use

related individuals (multigenerational pedigrees or sibpairs). An approach that has been applied successfully to the identification of regions in the genome that may house genes for complex polygenic diseases involves "genome scanning". This sets out to find disease loci without any *a priori* assumptions about their identity. Since diagnosis of these complex polygenic diseases is generally in middle age, when parents may be deceased, pedigree-based linkage analysis is often not possible. Instead, model-free approaches using affected sib pairs (ASPs) are more practical. Linkage to a disease is then indicated when a set of ASPs share more than the expected proportion of 50% of their alleles at a locus identical by descent (IBD).

In relation to blood pressure, as a quantitative trait, regions linked to variation in systolic blood pressure in general populations have been reported on chromosomes 6 and 16, and other loci not significant by suggested criteria have also been noted. In hypertensive patients examination of chromosome 17 has revealed a linkage region, but not a hypertension gene.

The tumor necrosis factor (TNF) receptor 2 (TNF-R2) gene (formerly *TNFR2*, now renamed tumor necrosis factor receptor superfamily member 1B, *TNFRSF1B*), has not previously been considered in the underlying genetic cause of these polygenic conditions. However, recent research has pointed to effects, involving TNF-R2, on insulin receptor signalling, plasma lipids, blood pressure, leptin, glucose and other factors of interest in cardiovascular disease and diabetes. The present inventor therefore decided to carry out case-control studies involving a polymorphism in *TNFRSF1B* in CAD, hypertension and NIDDM, and also to perform ASP linkage analysis of the chromosome on which *TNFRSF1B* resides, namely chromosome 1. The latter involved a microsatellite scan of chromosome 1, which represents 10% of the genome (~300 cM) and is gene-rich.

TNF-R2 (alias p75/80) has a similar ligand-binding domain as TNF-R1 (alias p55/60), but each differ markedly in cytoplasmic domains, indicating usage of distinct signal transduction pathways. TNF-R2 synergizes with TNF-R1, possibly by sequestering TNF-α due to its higher affinity and faster dissociation, and "passing" it to TNF-R1. Each receptor is expressed by most cells, but at different densities, and can be regulated independently. TNF-α can induce marked upregulation of TNF-R2 mRNA with little or no change in TNF-R1. This is followed by rapid shedding of the extracellular domain to give plasma soluble (s) TNF-R2 which neutralizes TNF at high concentrations, but, when low, preserves TNF activity and increases long-term effects. Enhancement by TNF-R2

of TNF-R1 promotes NF-κB activation and apoptosis. TNF-R2 also has independent effects that occur later, are of a long-term nature, and include cell proliferation. In addition, it is TNF-R2 that mediates strong stimulation by the transmembrane (pro) form of TNF.

Disclosure of Invention

A method of determining a predisposition in a subject to a complex polygenic disease, the method including assaying chromosome 1 for a genetic marker indicative of a predisposition to the complex polygenic disease.

The complex polygenic disease may be a cardiovascular disease. The complex polygenic disease may be coronary artery disease, non-insulin-dependent diabetes mellitus, neuropathy in NIDDM, essential hypertension or hyperlipidemia.

The method may include assaying for a genetic marker indicative of predisposition to CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia on chromosome 1, wherein detection of the genetic marker in the subject is indicative of predisposition to CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia.

The region of chromosome 1 of particular interest is 1p36.2.

Preferably, the genetic marker is situated in or near the gene (*TNFRSF1B*) encoding tumor necrosis factor receptor 2 (TNF-R2). More preferably, the marker is *D1S2834* or a closely related marker situated in the vicinity of *D1S2834* and indicative of predisposition to CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia.

Assaying of chromosome 1 for the genetic marker can be by any suitable means. Typically, polymerase chain reaction (PCR) is used utilising complementary primer pairs spanning at least a portion of the genetic marker of interest. PCR products are then separated by electrophoresis and analysed.

PCR primers found to be particularly suitable are as follows:
Forward primer, 5'-GTG ATC TGC AAG ATG AAC TCA C-3' (SEQ. ID. No:2);
Reverse primer, 5'-ACA CCA CGT CTG ATG TTT CA-3' (SEQ. ID NO:3).

Use of these primers in PCR will give bands of 367, 369, 371, 373 and 375 bp for alleles *CA13*, *CA14*, *CA15*, *CA16*, *CA17*, respectively.

It will be appreciated that other primers directed to the general *TNFSF1B* region of interest would also be suitable, for example, the primers directed to the *TNFRSF1B* intron 4 polymorphic region may be:

Forward primer, 5'-AAT CTG TGT GTG TGC ATG TG-3' (SEQ. ID. NO.:4)
Reverse primer, 5'-CTT GGT CAA CCA CTC AGA C-3' (SEQ. ID. NO: 5)
Use of these primers in PCR will give bands in the vicinity of 130 bp for alleles *CA13*, *CA14*, *CA15*, *CA16*, *CA17*, respectively.

In a second aspect, the present invention consists in a method of treating or affecting the onset or manifestations of CAD, NIDDM, neuropathy in NIDDM. essential hypertension or hyperlipidemia in a human subject, the method including manipulating the expression of *TNFRSF1B*or activity of expressed TNF-R2 in the subject such that CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia is prevented or reduced.

The finding by the present inventor that subjects with CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia, or predisposed to acquiring CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia, have alterations to the gene encoding TNF-R2 may be utilised to treat or prevent onset of CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia. If the expressed TNF-R2 has altered functionality resulting in CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia in these subjects, or if altered shedding of TNF-R2 from the membrane of the cell in which TNF-R2 is expressed is responsible for the CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia, or if overexpression of the gene *TNFRSF1B* is responsible for the CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia then treatments aimed at the expression or action of the altered TNF-R2, or of the overexpressed TNF-R2, or treatments directed at the factors that control expression of the gene *TNFRSF1B* or the processing of its transcribed mRNA, or at the factors involved in release into the circulation of the soluble fragment (sTNF-R2) from the membrane-bound receptor may be devised. Alternatively, it may be possible to directly alter or repair DNA in or near the TNF-R2 gene (*TNFR2*) by gene therapy, for example, such that the subject expresses a favourable level of TNF-R2 or, if the causative genetic variant is responsible for one or other amino acid change(s) in the encoded protein, a form of TNF-R2 predisposing to normal coronary arteries, normal insulin resistance, normal glucose tolerance, normal blood pressure, normal cholesterol levels or normal neural function in diabetic nephropathy.

In a third aspect, the present invention consists in the use of a genetic marker situated in or near the gene encoding tumor necrosis factor receptor 2 (TNF-R2) as a screening tool for the detection of predisposition to CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia in a human subject.

Preferably, the marker is a (CA)-repeat polymorphism located in intron 4 of *TNFRSF1B* (SEQ. ID. NO:1) or the microsatellite *D1S2834* or a closely related marker situated in the vicinity of *TNFRSF1B* or *D1S2834* and indicative of predisposition to CAD, NIDDM, neuropathy in NIDDM, essential hypertension or hyperlipidemia

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and accompanying drawings.

Brief Description of Drawings

Figure 1 shows an example of ABI 377/Genotyper result from which *TNFR2* genotypes were determined. Peaks represent fluorescently-labeled PCR products obtained by amplification of a region of intron 4 of *TNFR2* that contains a dinucleotide repeat polymorphism. PCR primers were: forward - 5'-GTG ATC TGC AAG ATG AAC TCA C-3' (HEX-labeled); reverse- 5'-ACA CCA CGT CTG ATG TTT CA-3'. Each 25 μl PCR mix contained 50 ng genomic DNA, 5.5 nmol each primer, 0.25 mmol/L each dNTP, 1 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.7 mmol/L $MgCl_2$. The PCR thermal cycler was set to the following program: an initial step at 95°C for 12 min, then 10 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 12 min, followed by 15 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, then 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, finishing with a step at 72°C for 30 min. Shown are: uppermost result, 267/271 bp heterozygote; middle, 271 bp homozygote; bottom. 267/271 bp heterozygote.

Figure 2 shows hypertension linkage results. Part (a) of Figure 2 shows two-point linkage data for significance region at 1p36.2 and map positions of relevant genes. The diagram on the left of Figure 2 part (b) shows placement of genes (on left of vertical line) on chromosome 1 map described by the markers used (shown on the right of vertical line). Vertical bars indicate the support interval for genes that have not been mapped precisely. (Map information was derived from the Cedar Genetics database.) The gene symbols that appear are as follows: *NPPA* is atrial natriuretic factor, *NPPB* is brain natriuretic factor, *CLCNKA* is chloride channel A. *CLCNKB* is chloride channel B, *CLCN6* is another chloride channel, and PGD is phosphoglycerate dehydrogenase. Part (b) of Figure 2 shows results for multipoint linkage analysis of chromosome 1 by the program MAPMAKER/SIBS. The upper panel of Figure 2 part (b) shows percentage information content. The middle panel of Figure 2 part (b) shows that multipoint maximum likelihood statistic (MLS) calculated under the assumption of no dominance variance using weighted sibships. The MLS peak (at *TNFRSF1B* marker) for unweighted sibships was 3.1. The lower panel of Figure 2 part (c) is an exclusion map calculated under the assumption of dominance variance for a hypothetical locus with relative risk to a sib (lambda-S: λ_s) of 1.6 and exclusion threshold of Lod = -2 being indicated.

Figure 3 shows the effect of the most common allele, *CA15*, of *TNFRSF1B* on plasma sTNF-R2 in normotensive (open bars) and hypertertesnive (hatched bars). Presence of allele other than *CA15* is indicated by "-". Hypertensive subjects who were homozygous for allele *CA15* had elevation in plasma sTNF-R2 when compared with hypertensive subjects heterozygous for *CA15* or who lacked a *CA15* allele. (In contrast, corresponding analyses of data for allele *CA16* revealed that sTNF-R2 was not elevated in any of the hypertensive subjects.) In the normotensive subjects, slight tracking of absence of the *CA15* allele with elevation in sTNF-R2 was seen, with levels being on average lower than those seen in the hypertensive *CA15/CA15* genotype group.

Figure 4 shows the effect of most common allele, *CA15*, and next most common allele, *CA16* on plasma lipids. In each case any allele other than the one shown is indicated by "-". These results suggest a genotypic effect on plasma total cholesterol (C) and high density lipoprotein cholesterol (HDL-C) that involves *TNFRSF1B*.

Figure 5 shows effect of *TNFRSF1B* intron 4 genotype on apo A-I in CAD patients. The *CA16* allele tracked with elevation in apo A-I ($F = 4.76$, $P < 0.0001$, which remained at the $P < 0.0001$ level after Bonferroni correction).

Figure 6 shows the effect of *TNFRSF1B* genotype on glycosylated hemoglobin (HbA1c) in NIDDM patients. The *CA16* allele tracked with lowering of HbA1c ($F = 4.81$, $P = 0.009$, which remained after Bonferroni correction: $P = 0.026$).

Figure 7 shows the new genetic findings from the present work in the context of existing knowledge. It illustrates the effect of *CA15* allele of *TNFRSF1B* genotype in stimulating plasma sTNF-R2, lipids and blood pressure, and how lack of response in individuals with *CA16* allele may result in less sTNF-R2 shedding, less TNF inhibition, and thus more vasodilatation (hypotensive).

Modes of Carrying Out the Invention

The outcome of a scan of chromosome 1 in hypertensive ASPs revealed a linkage in the vicinity of the tumor necrosis factor receptor 2 gene (*TNFSF1B*). As a result, association studies were conducted in various patient groups of a marker in *TNFRSF1B* and this showed significant associations with CAD, NIDDM, neuropathy in NIDDM, hypertension, blood pressure, sTNF-R2, hyperlipidemia, apo A-I or Hb1c.

METHODS

Hypertensive Subjects

The subjects were recruited from eastern Australia, mainly Sydney, by public advertising. Criteria were systolic/diastolic blood pressure of >140/95 mm Hg prior to treatment, lack of diabetes, heart or renal disease, and Anglo-Celtic ancestry. The study had ethical approval and all subjects gave informed consent.

Subjects for linkage study: The linkage study involved 200 ASPs (from 120 sibships that included 18 trios, 7 quartets and 1 quintet, and which were weighted by the standard method of Hodge). This number has been deemed sufficient for 90% power to show linkage at the Lod = 2 ($P = 0.001$) level in a complex, polygenic disease with multiple weak contributing loci (e.g., relative risk to a sibling, λ_s, values of ~1.6). Characteristics (mean ± SD) were: pretreatment systolic/diastolic BP, 171 ± 24 / 103 ± 10 mm Hg; age, 61 ± 10 years; age at disease onset, 43 ± 13 years; BMI, 27 ± 5 kg/m^2; male:female ratio, 0.35:0.65.

Subjects for hypertension association study: The hypertension association study involved a well-studied cohort of up to 133 hypertensive patients whose parents both had hypertension and up to 197 normotensive controls whose parents had normal blood pressure past the age of 50 years. Since only 1 in 10

hypertensive patients have two affected parents, this group was, in effect, drawn from >1000 hypertensive cases. Demographic parameters for each group resembled those we have described previously. For such genetically selected hypertensives, pre-treatment systolic/diastolic blood pressure, 175 ± 24 / 109 ± 18 mm Hg. mean age, 52 ± 12 years, and age at disease onset, 32 ± 10 years, demonstrated moderate to severe, early onset disease. The hypertensive and normotensive groups had similar body mass index (BMI) (27 ± 5 vs 26 ± 4 kg/m^2, respectively), but plasma lipids were elevated, as is commonly observed in patients with hypertension. Male:female ratio was 0.46:0.54.

Coronary Artery Disease Subjects

The coronary artery disease (CAD) association study involved 769 Caucasian patients referred to the Eastern Heart Clinic at Prince of Wales Hospital, Sydney for coronary angiogram, with a provisional diagnosis of CAD. Recruitment was confined to patients aged less than 65 y. Each patient's medical history was obtained using a questionnaire with standardized choices of answers to be ticked during the interview and DNA samples were extracted from venous blood collected from each patient by standard venepuncture of the antecubetal fossa. The severity of CAD was determined by the number of significantly stenosed coronary arteries. Each angiogram was classified as revealing either normal coronary arteries or having no coronary lesion with ≥ 50% luminal stenosis or as having one, two, or three major epicardial coronary arteries with more than 50% luminal obstructions. An angiographically normal coronary artery was defined as smooth contour and no luminal stenosis. Characteristics of the CAD patients (means only) were: age, 56 y; male:female, 0.74:0.26; smoking dose (pack/y), 29 (male), 14 (female); waist:hip, 0.98 (male), 0.86 (female); BMI, 28 kg/m^2; total cholesterol, 5.5 mmol/L (male), 5.7 (female); triglycerides, 2.2 (male), 1.9 (female); high density lipoprotein (HDL) cholesterol, 0.99 (male), 1.27 (female); low density lipoprotein (LDL) cholesterol , 3.5; total/HDL, 5.8 (male), 4.7 (female); apolipoprotein A-I, 0.88 g/L (male), 1.02 (female); Apo B, 0.99 (male), 0.93 (female); lipoprotein(a), 305 (male), 350 (female); glucose, 6.4 (male), 6.3 (female); fibrinogen, 3.6 (male), 3.9 (female). Controls and their characteristics have been described previously.

Non-insulin-dependent Diabetes Mellitus Subjects

The diabetes association studies involved 357 white Australian NIDDM patients of mean age 62 ± 12 SD years (male:female, 0.55:0.45) who attended

the Diabetes Centre at the Prince of Wales Hospital, Sydney regularly during May 1996 to May 1997. They were diagnosed according to the National Diabetes Data Group criteria. Complications, such as nephropathy, based on both clinical symptoms and biochemical parameters were assessed by endocrinological examination. Written consent was obtained from all the patients involved in the study, which was approved by the Ethics Committee of the University of New South Wales.

Glycemic control was assessed by glycosylated haemoglobin (HbA1c) determination using ion exchange HPLC. Serum creatinine and urinary albumin concentrations were determined by clinical chemistry. Microalbuminuria was diagnosed as the first urine sample collected in the morning with urinary albumin > 3 but < 30 mg/mmol creatinine.

Plasma Assays

Plasma sTNF-R2 was measured using a MEDGENIX enzyme amplified sensitivity immunoassay (BioSource Europe S.A., Fleurus, Belgium). For the samples from hypertensive patients and controls, plasma lipids (mmol/L) were determined by a Reflotron Plus (Boehringer Ingelheim, Germany). For the coronary artery disease and diabetic patients, total cholesterol, high density lipoprotein cholesterol (HDL) and triglyceride concentrations were measured by the Clinical Chemistry Department at the Prince of Wales Hospital using standard enzymatic methods. Values for low density lipoprotein (LDL) cholesterol were calculated from the Friedewald formula. Levels were not calculated in those patients whose triglyceride concentration was > 4.52 mmol/l since the formula then becomes inaccurate. In diabetics, to assess glycaemic control, glycosylated hemoglobin (HbA1c) was measured by ion exchange HPLC (Bio-Rad Variant, Anaheim, CA, USA; normal range 4.4-6.4%; inter-assay coefficient of variation 2.8%). Serum creatinine and urinary albumin concentrations were determined by clinical chemistry. Units were: apo A-I: mg/L; cholesterol: mmol/L; creatinine: mmol/L, glycosylated hemaglobulin: %.

Genotyping

Microsatellite markers on chromosome 1

Patient DNA of ASPs was extracted from leukocytes using a Qiagen kit. Genotypes were determined for 24 microsatellite markers in panels 1 and 2 of the Applied Biosystems Inc (ABI; Foster City, CA, USA) PRISM™ Linkage Mapping Set by PCR. The forward primer of each pair was labeled at the 5'-end with 6-carboxyfluorescein or its tetrachlorinated or hexachlorinated analogues (FAM,

TET or HEX). PCR conditions, which had been optimized for each primer pair, were as recommended by the supplier. The PCR products were electrophoresed on an ABI 377 automated sequencer and genotypes were assigned using ABI Genotyper™ software. Six additional markers (*D1S2667*, *D1S434*, *D1S2834*, *TNFRSF1B*, *D1S2728*, *D1S436*), selected from the Genome Database (http://gdbwww.gdb.org/gdb/gdbtop.html), were deployed subsequently. Primers for these were synthesized by Bresatec (Adelaide, South Australia), the forward primer being labeled with either HEX or TET. The sequences of the primers for amplification of microsatellite markers that were subsequently found to form a hypertension linkage peak can be found in the SEQUENCE LISTINGS section later. Their sequence identification numbers are as follows: *D1S228* forward primer (SEQ. ID. NO.: 6), D1S228 reverse primer (SEQ. ID. NO.: 7); *D1S2834* forward primer (SEQ. ID. NO.: 8), D1S2834 reverse primer (SEQ. ID. NO.: 9); *TNFRSF1B* intron 4 microsatellite marker forward primer (SEQ. ID. NO.: 2), *TNFRSF1B* intron 4 microsatellite marker reverse primer (SEQ. ID. NO.: 2); *D1S2728* forward primer (SEQ. ID. NO.: 10), *D1S2728* reverse primer (SEQ. ID. NO.: 11); *D1S436* forward primer (SEQ. ID. NO.: 12), *D1S436* reverse primer (SEQ. ID. NO.: 13). Map intervals for the various markers can be found at http://cedar.genetics.soton.ac.uk/pub/chrom1/map.html. An example of ABI 377/Genotyper result for detection of the *TNFRSF1B* genotypes by primers SEQ. I.D. No.: 2 and SEQ. I.D. NO.: 3 is shown in Figure 1. An alternative primer set was also designed for detection of the *TNFRSF1B* intron 4 microsatellite polymorphism and is shown in the sequence listings section as SEQ. I.D. NO.: 4 and SEQ. I.D. NO.: 5.

Other TNFRSF1B *polymorphisms*

Besides the microsatellite marker in intron 4 of *TNFRSF1B* (alleles *CA13*, *CA14*, *CA15*, *CA16*, *CA17*), we also developed methods for genotyping putative variants at -353(22), -1120 and in exon 6 of *TNFRSF1B*. Screening of 70 chromosomes revealed that only the exon 6 variant (T685G: Met198Arg) was real (the others being sequencing errors in the literature or rare variants). The exon 6 polymorphism was detected by PCR-RFLP with primers 5'-CCG TGA ATG AGC CCA G-3' (SEQ. ID. NO.: 14) and 5'-CAG AAG GAG TGA ATG AAT GAG-3' (SEQ. ID. NO: 15), 1.5 mmol/L $MgCl_2$, and 36 PCR cycles. The *G* allele (344 bp) was not cut by *Hsp*92II, but the *T* allele was (yielding 109 and 235 bp bands).

Chloride channel gene polymorphisms

Polymorphisms were also tested in other genes that localize within band 1p36.2. One of these was a marker in the chloride channel gene *CLCNKA*. Detection of this involved single stranded conformational polymorphism (SSCP) analysis, a technique capable of detecting single base pair changes. It used PCR to amplify the region of interest and PCR products are run on an electrophoretic gel. Differences in migration are indicative of sequence variation. *CLCNKA* is highly homologous to *CLCNKB*, and although the human *CLCNKA* cDNA sequence was available, primers used to amplify *CLCNKA* exon 13, where a variant is said to exist, would also amplify *CLCNKB* exon 13. Designing of new primers was limited by the lack of published information on the intronic regions of *CLCNKA*. Therefore, primers reported by others were used, i.e., forward: 5'-CCT CAG GGA TGG AGG GCT GTG-3' and reverse: 5'-CAC GAC ATT GCC CAC GCA GCA G-3'. PCR involved 10 cycles of 1 min each 94°C, 65°C and 72°C, followed by 15 cycles for 1 min each 94°C, 60°C and 72°C, then 20 cycles for 1 min each at 94°C, 58°C and 72°C, finishing with a step at 72°C for 30 min. The PCR mixture consisted of 24 µl of 3 pmol each primer, 0.2 mmol/L of each dNTP, 0.1 U of Amplitaq Goldô (Perkin-Elmer, Norwalk, CT), 2 mmol/L $MgCl_2$, 10 x Buffer and ddH_2O. To distinguish *CLCNKA* from *CLCNKB* in the same PCR involved digestion with *Hin*fI (New England BioLabs) at 37°C for 3 h. Bands were visualized by ethidium bromide staining after electrophoresis on a 1.5% high resolution agarose gel, and then purified using gel extraction kit (BresaGen). The purified *CLCNKA* PCR product was then subjected to SSCP and sequencing using a commercial service (Australian Genome Research Facility, Brisbane, Australia). DNA sequencing involved initial amplification of the exon 13 region using the following primers: *CLCNKA* and *CLCNKB*: forward: 5'-CCT CAG GGA TGG AGG GCT GTG-3'; reverse: 5'-CAC GAC ATT GCC CAC GCA GCA G-3', with *Hin*fI being used to identify which was *CLCNKA*. The PCR product was extracted from the gel, cloned and sequenced. We found a polymorphism G1339A (Ala470Thr) in exon 13 of *CLCNKA*. This was in the transmembrane region and could have the potential to affect conductance through the membrane. We also found a C1389A polymorphisms (Pro463Pro: silent variant).

Conditions for SSCP were modified from the method of Orita *et al.* Ten microlitres of formamide gel loading buffer, which consisted of 40 mM EDTA, 95% formamide and 0.05% Orange G (Sigma-Aldrich), was added to

10 µl of purified PCR product, heat shocked for 4 min at 95°C. and then immediately put on ice for 2 min. All of the 20 µl was electrophoresed at 16 mA for 6 h on a 16% non-denaturing polyacrylamide gel. The bands were visualized using silver staining of the PAGE gel, which was fixed in 10% ethanol for > 5 h, washed in MilliQ distilled water 3 times, then put in 1% HNO_3 for 3 min, rinsed again in MilliQ water 3 times and then silver stained in 10% $AgNO_3$ for at least 30 min. Extensive rinsing in MilliQ water enhanced the resolution of bands on the gel after developer. The gel was rinsed in MilliQ water to wash off excess silver grains and then placed into the developer, 15% $NaCO_3$ and 0.05% formaldehyde, for 5 min. Band resolution was dependent on the amount of time in the developer. This reaction was stopped by rinsing in MilliQ water 3 times and in 3% acetic acid for at least 10 min. The gel was fixed in 10% ethanol overnight. Thorough rinsing of the gel after acetic acid treatment was crucial for the storage of the gel, which was dried using Gel Drying Kit (Promega). A control sample was loaded in each gel run to recognize different SSCP patterns. For each different SSCP pattern, the purified PCR product was cloned using pGMT-easy kit (Promega) and sequenced (Australian Genome Research Facility, Melbourne, Australia) to identify the triallelic polymorphism of *CLCNKA* exon 13 mentioned by Simon *et al* in a report in 1997, but who did not provide details. The SSCP patterns were then assigned to each subject.

Atrial natriuretic factor receptor gene polymorphism

Restriction fragment length polymorphism (RFLP) analysis was used to detect a *XhoI* restriction fragment length polymorphism of *NPPA*. The details of the method used have been published previously by the present investigators.

Statistical Analyses

Evidence for linkage was assessed by non-parametric ASP methods. Since the relative power and behaviour of different standard nonparametric test statistics used for complex traits are known to vary, we applied several of the better methods to test for discordant allele sharing by descent (IBD) or by state (IBS). One was SPLINK (version 1.08), which generates IBD estimates using all sibs in a sibship under the constraints imposed by linkage (possible triangle restriction: $z[1] < 0.5$ and $z[0] < 0.5 \times z[1]$, where z[1] and z[0] are sharing of 1 and 0 alleles IBD, respectively) and compares the IBD distribution to that expected under no linkage. Both weighted and unweighted SPLINK was applied,

where number of weighted sibpairs in a family = number of unweighted sibpairs - (2 x number of affected siblings). Two-point linkage was also performed by IBS χ^2, which compares observed alleles shared IBS at a marker with what would be observed randomly using contingency table analysis. In addition, MAPMAKER/SIBS which restricts maximization of the likelihood ratio of Risch to within the possible triangle, was used to generate multipoint maximum Lod score (MLS) values at 1 cM intervals, as well as produce information content and exclusion map. For MAPMAKER/SIBS, allele frequencies were estimated using the affected families weighted by number of founders in each pedigree. The significance thresholds set for acceptance of linkage were as recommended, with a Lod of 3.2 advocated as the 5% significance level for a genome-wide scan.

For the hypertension association study, StatViewô (Abacus Concepts, Berkeley, CA, USA) was used for various statistical tests. Determination of linkage disequilibrium between polymorphisms involved analysis of haplotype frequencies in the largest group, as described. For the CAD and NIDDM data analyses, associations between genotype distribution and categorical variables involved Pearson's χ^2 test. Assessment of the effect of genotypes on continuous variables was by 1-way ANOVA. In the study of CAD patients, all the continuous variables are presented as mean ± SE. Hardy-Weinberg equilibrium was assessed by chi-squared (χ^2) analysis. A contingency table χ^2 analysis was employed to estimate the contribution of the polymorphism to the occurrence and severity of CAD, and to evaluate relationships between the genotypes and other medical conditions including myocardial infarction, family history of CAD, and hypertension. Logistic regression analysis (stepwise linear model) was used to assess the association between the polymorphism and CAD, with other known risk factors being controlled for. One-way analysis of variance (ANOVA) was used to compare parameters across genotypes. In the NIDDM subjects a logistic regression model was employed to ascertain the independent association between the genotypes and microalbuminuria or nephropathy while the other variables, including age, sex, duration of diabetes and methods of treatment were controlled for in the model. Two-tailed *P* values were reported and all statistical analyses were conducted using SPSS version 9.0 for Windows (SPSS Inc, IL).

RESULTS

Linkage study

The primary scan involved ABI markers for chromosome 1. One marker (*D1S228*) in the ABI set used indicated suggestive linkage with HT, so we proceeded to test additional markers nearby, namely, *D1S2667*-3.8 cM-*D1S434*-2.7 cM-*D1S228*-2.3 cM-*D1S2834*-4.7 cM-*D1S2728*-4.6 cM-*D1S436*, and a microsatellite in intron 4 of *TNFRSF1B*, which is 0.1-3.1 cM centromeric of *D1S2834* according to the Cedar Genetics database, and 150-440 kb from *D1S434* according to other published data. Two-point linkage scores indicated a peak in which *D1S228*, *D1S2834* and *TNFRSF1B* (heterozygosity 75%, 69% and 58%, respectively) formed the apex (Figure 2). Allele sharing for *D1S2834* and *TNFRSF1B* (64.2% and 63.8%, respectively) significantly exceeded 50%. From IBD estimates using SPLINK, the *TNFRSF1B* locus contributed to 37% of familial involvement in hypertension using a non-additive model and 27% using an additive model. Multipoint linkage analysis using MAPMAKER/SIBS gave peak Lod score of 3.1 (unweighted) and 2.5 (weighted) (Figure 2). An exclusion map (Figure 2) did not support hypertension linkage for markers telomomeric of *D1S228* and centromeric to *D1S436* (angiotensinogen, at q42, is thus excluded, as detailed elsewhere in two recent reports, one of which was from from the present inventor's Laboratory. Figure 2 part (a) left hand side shows marker and gene locations in the vicinity of the region showing highest significance.

Association Studies

Hypertension association study

TNFRSF1B *intron 4 variant chi-squared analysis:*

Allele frequencies for the *TNFRSF1B* intron 4 polymorphism is the hypertensive and control normotensive groups are shown in Table 1. A significant difference was observed.

Table 1. Association of intron 4 variant in *TNFRSF1B* with hypertension.

		Total alleles on all chromosomes (proportion in brackets)						
Group	n	*CA13*	*CA14*	*CA15*	*CA16*	*CA17*	χ^2	*P*
HT	133	41 (0.15)	12 (0.05)	140 (0.53)	68 (0.26)	5 (0.02)	11.7	0.020
NT	173	69 (0.20)	4 (0.01)	199 (0.58)	71 (0.20)	3 (0.01)		

(In Table: HT. hypertensive subjects (offspring of 2 HT parents); NT, healthy control subjects who were the offspring of 2 parents who were NT past the age of 50 y)

TNFRSF1B *exon 6 variant and haplotype analysis:*

Besides the intron 4 marker, genotypes for a variant in exon 6 were determined. Each of these polymorphisms by itself was in Hardy-Weinberg equilibrium. The markers were found to be in linkage disequilibrium with each other, the *G* allele of the exon 6 variant being associated with alleles *CA13* and *CA14* of the intron 4 variant (D' = 88%, $P < 0.001$). Allele frequencies of the intron 4 variant in the normotensive group were similar to values reported for 78 unrelated probands from Centre d'Etude du Polymorphisme Humaine pedigrees (viz. 0.19, 0.02, 0.54, 0.24 and 0.01, respectively). Frequency of the *T* allele of the exon 6 variant in the normotensive group was 0.74 (n = 197) and in the hypertensive group was 0.75 (n = 123). Traditional haplotype analysis by excluding the *GG* genotype revealed significant excess of *CA16/T* haplotypes in the hypertensive group (n = 64 with and 144 without this haplotype) compared with the normotensive group (n = 75 and 273): i.e., frequencies = 0.31 and 0.22 (χ^2 = 5.9, P = 0.015).

Plasma soluble TNF-R2 protein vs blood pressure and genotype:

Plasma sTNF-R2 (mean ± SE; ng/ml) correlated with diastolic (r = 0.28, P = 0.0001) and systolic (r = 0.23, P = 0.0002) blood pressure (pretreatment values in the case of hypertensives). sTNF-R2 was 3.9 ± 0.2 in hypertensives and 3.2 ± 0.07 in normotensives (n = 67 and 194, respectively; $P < 0.0001$). In Figure 3, shown is the effect of the most common allele, *CA15*, of *TNFRSF1B* on plasma

sTNF-R2, where presence of allele other than *CA15* is indicated by "-". Table 2 is an updated version of the data in Figure 3. It was noted that sTNF-R2 was 34% higher in *CA15* homozygotes cf. *CA15* heterozygotes and 24% higher than for absence of *CA15* allele. The difference in sTNF-R2 in *CA15* homozygotes between hypertensives and normotensives was significant ($F = 25$, $P < 0.0001$) as was 2-way ANOVA of hypertensives vs normotensives for comparison of sTNF-R2 across genotypes with and without the *CA15* allele ($F_{2df} = 16$, $P < 0.0001$) (Table 2). It should be noted that in Table 2 data for normotensives show that the effect of *CA15* genotype on sTNF-R2 was in the opposite direction as in the hypertensives, i.e., possession of a *CA15* allele was associated with reduction in sTNF-R2. Data in Table 2 are an expanded data set from values shown in Figure 3 and as a result *P* value for 2-way ANOVA in Table 2 is more significant than *P* value in Figure 3.

Table 2. Plasma soluble TNF-R2 (ng/ml) in hypertensives (HT) with 2, 1 or no *CA15* alleles (upper set of data) and in normotensives (NT) with 2, 1 or no *CA15* alleles (lower set of data).

	CA15/CA15	*CA15/-*	*-/-*	F	*P*
HT	4.7 ± 0.5	3.5 ± 0.2	3.8 ± 0.4	2.5	0.092
	(n = 16)	(n = 21)	(n = 19)		
NT	2.99 ± 0.11	3.32 ± 0.10	3.44 ± 0.15	3.6	0.029
	(n = 63)	(n = 78)	(n = 41)		

(2-way ANOVA: $F = 16.5$, $P < 0.0001$)

In contrast to allele *CA15*, in the case of allele *CA16*, sTNF-R2 was not elevated (3.4 ± 0.7, 3.8 ± 0.4, 4.2 ± 0.3 for *CA16/CA16*, *CA16/-*, *-/-*; n = 7, 19, 33, respectively; $F = 0.71$, $P = 0.49$), in fact was slightly lower for patients with the CA16 allele, influenced no doubt by the higher sTNF-R2 of *CA15*, which makes a major contribution to the "-" component in the *CA16* analysis.

Pretreatment blood pressure was available for only 7 *CA16* homozygotes. However, there were 36 *CA16* heterozygotes with blood pressure values. Diastolic blood pressure of *CA16* heterozygotes was higher than for patients lacking this allele, viz. 116 ± 19 vs 106 ± 16 and 103 ± 8 for *CA16/-*, *-/-* and *CA16/CA16*, respectively (n = 36, 61, 7; $F = 4.7$, $P = 0.012$). Systolic pressures were similar. No blood pressure differences were seen in relation to presence of absence of *CA15* allele when comparing across genotypes.

A significant positive correlation was also seen between sTNF-R2 and age ($r = 0.24$, $P < 0.0001$), i.e., sTNF-R2 rose as patients got older (or their hypertension had been present for a longer time).

TNFRSF1B *genotype vs plasma lipids:*

Patients with hypertension are well known to exhibit hyperlipidemia as a characteristic feature. The *CA15* allele of *TNFRSF1B* was found to track significantly with elevation in total cholesterol, HDL-C and LDL-C in the hypertensive group (Table 3; Figure 4), but in the normotensive group nonsignificant tracking in the opposite direction was apparent. Figure 4 shows the effect of most common allele, *CA15*, and next most common allele, *CA16*. In each case any allele other than the one shown is indicated by "-".

Table 3. Plasma lipid values (mmol/L) in hypertensives with 2, 1 or no *CA15* alleles (upper set of data) or *CA16* alleles (lower set of data).

	Genotypes				
	CA15/CA15	*CA15/-*	*-/-*	F	*P*
n	30	50	23	-	-
Total-C	6.21 ± 0.22	5.74 ± 0.17	5.25 ± 0.22	4.3	0.016
Triglycerides	2.28 ± 0.32	2.44 ± 0.18	2.10 ± 0.33	0.40	0.67
HDL-C	1.31 ± 0.11	1.02 ± 0.07	1.03 ± 0.09	3.5	0.035
LDL-C	4.39 ± 0.17	3.82 ± 0.16	3.67 ± 0.31	3.0	0.052
	CA15/CA15	*CA15/-*	*-/-*	F	*P*
n	7	31	56	-	-
Total-C	5.05 ± 0.19	5.40± 0.18	6.05 ± 0.16	4.7	0.011
Triglycerides	2.04 ± 0.50	2.43 ± 0.25	2.28 ± 0.20	0.23	0.80
HDL-C	0.91 ± 0.10	0.95 ± 0.09	1.21 ± 0.07	3.7	0.027
LDL-C	3.46 ± 0.42	3.45 ± 0.22	4.28 ± 0.13	10.72	0.0024

In patients with the hypertension-associated allele (*CA16*) LDL-C showed a significant negative correlation with both systolic ($-r = 0.25$, $P = 0.030$) and diastolic ($-r = 0.35$, $P = 0.0017$) blood pressure. This could be an effect of elevated mortality of those with higher LDL-C.

The results in Table 3 and Figure 4 suggest a genotypic effect on plasma lipids that involves *TNFRSF1B*. In overweight hypertensive patients (BMI > 25 kg/m^2) difference in LDL-C values across genotypes was more marked than in the

group as a whole: 4.64 ± 0.33, 3.65 ± 0.24 and 1.75 ± 1.15 for *CA15/CA15*, *CA15/-*, *-/-* (n = 10, 16, 2; $P = 0.0029$).

Chloride channel gene markers - association findings

We also tested polymorphisms in other potential candidate hypertension genes at 1p36.2. By single-stranded conformational polymorphism analysis it was determined that exon 13 of the chloride channel gene *CLNCKA* was polymorphic. The G1339A (Ala470Thr) variant was not associated with hypertension: minor (A) allele: 0.11 and 0.13 in hypertensive and normotensive groups, respectively (n = 104 and 181; $\chi^2 = 3.3$, $P = 0.19$). For the C1389A (Pro463Pro) variant, minor (A) allele was similar, 0.17 and 0.18, in the respective groups (n = 71 and 118).

Atrial natriuretic factor gene marker - association findings

Examination of *NPPA* for a *Xho*I RFLP in 66 hypertensives and 75 normotensives showed no association with hypertension: minor allele frequency in each group = 0.50 and 0.46, respectively.

Coronary artery disease association study

Table 4 shows total alleles on all chromosomes for the CAD population and the healthy control population.

Table 4. Association of *TNFRSF1B* intron 4 polymorphism with CAD.

		Total alleles on all chromosomes (proportion in brackets)						
Group	n	*CA13*	*CA14*	*CA15*	*CA16*	*CA17*	χ^2	*P*
CAD	769	273 (0.18)	43 (0.03)	699 (0.46)	491 (0.32)	12 (0.01)	24.5	< 0.0001
Control	173	69 (0.20)	4 (0.01)	199 (0.58)	71 (0.20)	3 (0.01)		

As in the hypertensives, *CA16* frequency was elevated and *CA15* suppressed in the patient group. Comparing the major allele, *CA15*, between CAD and control (0.46 vs 0.58) gave $\chi^2 = 13.9$, $P = 0.001$. Comparing *CA16* frequency

in CAD (0.32) vs control (0.20) gave $\chi^2 = 24.3$, $P = 0.00001$. The high significance in Table 4 is maintained even if the analysis was only confined to those with angiographically demostrable coronary lesions. allele frequencies being 0.18, 0.03, 0.45, 0.33. 0.01 for *CA13. CA14. CA15. CA16, CA17,* respectively. and are highly significant for *CA15* allele comparison ($\chi^2 = 22.5$. $P < 0.0001$)and for *CA16* allele comparison between CAD and control ($\chi^2 = 20.5$, $P < 0.0001$). Comparing to healthy controls. the risk for *CA15* allele to have CAD was 0.61 (95%CI: 0.48 - 0.78): the risk for *CA16* allele to have CAD was 1.89 (95%CI: 1.42 - 2.52).

There was no association with severity of CAD (number of diseased coronary vessels), nor BMI, waist:hip ratio or plasma cholesterol concentrations. However, there was a significant association between the presence of the *CA15* allele and plasma apo A-I concentration: 0.86 ± 0.027, 0.84 ± 0.018, 0.99 ± 0.032 mg/L for *CA15/CA15, CA15/-, -/-* (n = 180, 324, 228: F = 11.2, $P < 0.0001$). The apo A-I levels remained significantly higher in the -/- genotypes after Bonferroni correction when comparing *CA15/CA15* with *CA15/-* ($P < 0.0001$). The *CA16* allele tracked with elevation in apo A-I: 1.03 ± 0.049, 0.89 ± 0.025, 0.85 ± 0.018 for *CA16/CA16, CA16/-, -/-* (n = 112, 236, 384; F = 9.1, $P < 0.0001$) (Figure 5). The difference between the *CA16/CA16* and -/- remained significant after Bonferroni correction ($P < 0.0001$).

NIDDM association study

In 358 NIDDM patients *TNFRSF1B* intron 4 allele frequencies were 0.12, 0.03. 0.64, 0.12, 0.02 compared with 0.20, 0.01, 0.58, 0.20, 0.01 in healthy control group. Only allele *CA13* frequency was different (0.13 vs 0.20, respectively). The lower *CA13* frequency contributed to the value for χ^2 of 11.6 ($P = 0.003$) when NIDDM group was compared with control group. (In each of the various study groups, including the NIDDMs, we observed *very* rare alleles *CA10, CA11, CA18* and *CA20.* In each case data for *CA10* and *CA11* were combined with *CA13* and data for *CA18* and *CA20* were combined with *CA17.*) In other words, the major alleles that were the cause of the significant differences between data for hypertensive group and control, and between CAD group and control, did not differ for NIDDM vs control. Thus for *CA15/CA15, CA15/-, -/-* frequencies were 0.40, 0.45. 0.15 (n = 143, 162, 52).

No associations were found between the *CA15* allele and any of the complications of diabetes, including retinopathy, nephropathy, neuropathy, peripheral vascular disease, coronary heart disease and cerebrovascular disease.

Heterozygotes had higher HDL-C and HbA1c, and lower creatinine. Values for HDL-C were 1.07 ± 0.31, 1.22 ± 0.39 and 1.09 ± 0.30 in *CA15/CA15*, *CA15/-*, *-/-*, respectively ($P = 0.006$) and significance remained after Bonferroni correction ($P = 0.001$). However, whether this was a statistical artefact, or implies heterogeneity in diabetic complications, or different roles in diabetic complications for TNF-R2 in different tissues, cannot be easily explained.

For the *CA16* allele, frequencies of *CA16/CA16*, *CA16/-*, *-/-* were 0.03, 0.32, 0.65 (n = 10, 116, 232). HDL-C tended to be higher in *CA16/CA16* homozygotes (1.28 ± 0.21, 1.22 ± 0.36 and 1.11 ± 0.21, respectively; n = 9, 110, 218; F = 4.7, $P = 0.009$). The HDL-C levels remained significantly higher in the *-/-* genotypes after Bonferroni correction when comparing *CA16/CA65* with *CA16/-* ($P = 0.013$). After Bonferroni correction, only the difference between the *CA16/-* and *-/-* remained statistically significant ($P = 0.013$). The number for *CA16/C16* was too small to reach statistical significance, although the mean HDL-C level was highest in the *CA16/CA16* group. However, if *CA16* allele carriers (i.e., *CA16/CA16* + *CA16/-*) are compared to the non-carriers (*-/-*) the difference was significant ($P = 0.002$). HbA1c, a marker for hyperglycemic control, was lower in *CA16/CA16* (6.6 ± 0.3, 8.3 ± 0.2, 8.1 ± 0.1, respectively; n = 9, 102, 205; F = 5.0, $P = 0.007$) (Figure 6). The HbA1c levels remained significantly higher in the *-/-* genotypes after Bonferroni correction when comparing *CA16/CA16* with *CA16/-* ($P = 0.026$).

The *CA16* allele was significantly associated with neuropathy (Table 5). In a stepwise logistic regression model in which other factors including age, sex and duration of diabetes since diagnosis were controlled for, the *CA16* genotype still significantly predicted neuropathy. The OR for *CA16* carriers (of 1 or 2 alleles) was 2.1 (95% CI, 1.2-3.8) when compared with *-/-* individuals. The ORs for *CA16/CA16* when compared to *CA16/-* and *-/-* were 2.5 (0.6-11.2) and 4.8 (1.1-20.1), respectively. There was no association, however, with nephropathy or vascular complications.

Table 5. Association of *CA16* allele of *TNFRSF1B* intron 4 polymorphism with neuropathy in NIDDM.

		Genotype frequencies (fraction in brackets)				
Neuropathy	n	*CA16/CA16*	*CA16-*	-/-	χ^2	*P*
Yes	69	5	27	37		
		(0.07)	(0.39)	(0.54)		
					8.99	0.011
No	230	4	67	159		
		(0.02)	(0.29)	(0.69)		

DISCUSSION

The present inventor has found an essential hypertension locus at 1p36.2. The various ASP linkage analysis methods have their own particular advantages and disadvantages, so that consistent indications from several tests, as used here, provide some reassurance about the validity of the findings. Despite the absence of any requirement for information concerning penetrance, phenocopy, genetic heterogeneity or disease prevalence, allele sharing methods nevertheless depend on accurate estimates of allele frequencies. Reasonable estimates of allele frequencies can be obtained from a sample of approximately 200 ASPs by way of gene counting or maximum likelihood data. Because allele frequency estimates are necessary for reconstruction of parental haplotypes in order to determine inheritance of alleles, the ASPs themselves are preferred for allele frequency estimation. A recent simulation study involving a marker with 4 equally frequent alleles demonstrated that SPLINK gives the lowest false positive rate and greatest power to detect linkage for ASP data in the absence of parental genotypes. The higher Lod scores we observed for unweighted SPLINK would suggest that allele sharing is increased in larger affected families. We obtained, moreover, consistent findings by SPLINK, IBS χ^2 and MAPMAKER/SIBS in disclosing a linkage peak centred near *D1S2834* and *TNFRSF1B*. Significant probability values at adjacent loci at the Lod = 3, $P = 10^{-4}$ level and values ascending to these for adjacent markers, together with Baysian probabilities, genotype/phenotype observations, support from physiological data, and considerations for complex traits, support our linkage finding.

The linkage region lies within 1p36.2, where several genes of interest in hypertension have been localized, namely the renal chloride channel genes. *CLCNKA*, *CLCNKB* and *CLCN6*, natriuretic peptide genes, *NPPA* and *NPPB*, and tumor necrosis factor receptor 2 gene, *TNFRSF1B*.

CLCNKA and *CLCNKB* have the potential to influence blood pressure chronically, as demonstrated by Bartter's syndrome type III, which is caused by functional mutations in *CLCNKB* leading to hypotension, a feature of this condition. The 1000:1 support interval for *CLCNKA* and *CLCNKB* (Figure 2a) extends from *D1S507* to *D1S199*, and is centered at *D1S436*, where *D1S507* is 1.0 cM centromeric to *D1S2728*, i.e., is 5.7-16.5 cM from the apex of the peak indicated by the present linkage data. However, no association with hypertension was found for polymorphisms in exon 13 of *CLCNKA*.

In the case of *NPPA* and *NPPB*, not only are these genes able to influence blood pressure chronically, as shown by transgenic and knockout experiments, but plasma atrial natriuretic peptide is less responsive to a physiological challenge in subjects with a positive family history of hypertension. *NPPA* resides, however, between *D1S434* and *D1S2667* (Figure 2a), i.e., is 7.6 cM from the apex of the peak the present inventor observed. Moreover, association analyses proved negative for *NPPA* polymorphisms in hypertension in the present case-control groups, as well as in "four-corners" analyses by others of Caucasian subjects in Glasgow. Neither has any association been found for *NPPA* variants and salt-sensitivity. For *NPPB* the locus is in a similar region as the chloride channel genes discussed above (Figure 2a).

The 6-phosphogluconate dehydrogenase gene (*PGD*) has been linked to a 7 mmHg difference in diastolic pressure (viz. 72 vs 79 mmHg for *AA* and *AC*, respectively, for genotypes inferred from plasma PGD activity) in a study of 4 large Caucasian families (923 subjects aged > 8 years) ascertained by a hypertensive proband. *PGD* is 6.9 cM telomeric to the peak at *D1S2834*, residing between *NPPA* and *D1S434* (Figure 2a).

A linkage result applies to a much broader region than an association finding. This is because association studies test a polymorphism that, when it arose, showed complete disequilibrium with alleles of neighbouring linked markers, but which, after recombination over succeeding generations, has led to dissipation of the early linkage disequilibrium, such that the disease variant is associated only with the markers that are closest to it. The recombination events

and the number of generations elapsed between the present patients and the founder narrow the genomic region in disequilibrium to a small interval encompassing only the variant and nearby markers, i.e., to a region that is very much smaller than that which applies for a linkage result. A recent simulation study suggested moreover that linkage disequilbrium may extend only 3 kb and is never seen beyond 30 kb. Although this is not yet generally accepted, even being disputed by some experts, the present results are consistent with the possibility that a causative variant could reside in or near the 26 kb *TNFRSF1B* gene. Given the lingering uncertainties, linkage disequilibrium with a causative variant in a nearby gene cannot, however, be ruled out.

Linkage and association studies are complementary approaches that have different strengths and weaknesses. While association studies are more powerful than linkage studies in determining genetic causality, they encounter many problems, such as population stratification, making a positive result less reliable. On the other hand, a positive linkage result does not imply causality at that marker, as positive linkage could occur if a disease locus is within approx. ± 3 cM of the marker. If a locus is causative for a disease then both linkage and association should be shown, as we indeed have demonstrated in our study, which consequently involved two separate populations of HT patients. Another approach is the family based, transmission disequilibrium test (TDT). It is unfortunately difficult to perform robust TDTs for late onset diseases such as hypertension, since these require a heterozygous parent to be available for testing. The use of sib-TDTs would require the recruitment of much larger sibships. Moreover, the TDT is less powerful than case-control analysis, and much less powerful than the type of case-control approach we use of selecting patients with two affected parents.

A *prima facie* case can be made for *TNFRSF1B* in hypertension etiology. The present finding of a correlation of blood pressure with plasma sTNF-R2 most likely reflects a response of sTNF-R2 to elevation in plasma TNF in hypertensives. An elevation in TNF is known to occur later in the course of hypertension, possibly from vascular damage. It is also possible that TNF could be raised early in the course of the disease, since in hypertensive patients, antibodies to *Chlamidia pneumoniae* and *Helicobacter pylori* are more prevalent, and a microbiological or autoinflammatory basis for cardiovascular disease and NIDDM has been invoked. TNF can, via stimulation of inducible nitric oxide synthase (iNOS), induce apoptosis and vasodilation of vascular smooth muscle.

The shedding of sTNF-R2 by the plasma membrane of cells leads to binding to and inhibition of circulating TNF. This would serve to ameliorate in part the blood pressure lowering actions of TNF. Overall though, despite a rise in sTNF-R2 shedding, the response to TNF would be expected to be one of reduced blood pressure. The strong positive correlation of sTNF-R2 with both diastolic and systolic blood pressure is interpreted as indicating progressively greater TNF with higher and higher blood pressure, i.e., the greater the blood pressure, the more the vascular damage and other effects that result in release of TNF into the circulation and thus the greater the response in sTNF-R2 shedding to such raised TNF. TNF is also produced by renal tubules where it affects ion transport and serves to oppose pressor stimuli such as angiotensin II.

A decrease in the shedding response in sTNF-R2 to the presence of TNF would thus be expected to lead to elevation in blood pressure. Although we found no strong evidence to support such a hypothetic effect involving TNFRSF1B genotype, there was a suggestive effect involving the major allele (*CA15*). Patients (but not controls) who were homozygous for the *CA15* allele displayed higher sTNF-R2 than other genotypes. This is consistent with the hypothesis that patients with the *CA15/CA15* genotype respond by increased shedding of sTNF-R2. Moreover, the significantly higher plasma sTNF-R2 in hypertensives compared with control normotensives would appear to be contributed entirely by the *CA15/CA15* genotype. In contrast patients with the *CA16* allele had similar sTNF-R2 as patients lacking the *CA16* allele. As argued in the previous paragraph, higher sTNF-R2 is a marker for higher TNF and thus lower blood pressure. A reduced response to TNF (reduced sTNF-R2 shedding) would thus predispose to higher blood pressure. This may explain the association between the CA16 allele and hypertension. However, other factors influenced by *TNFRSF1B* genotype may also play a role in bringing about an elevation in blood pressure. Also, other, independent, genetic effects are expected to be necessary in order to produce the totality of the blood pressure elevation that is seen in a hypertensive individual.

The association of the *CA15* allele with elevation in sTNF-R2 and plasma cholesterol is consistent with the ability of TNF to increase plasma lipids, which involves stimulation of hepatic lipid synthesis and secretion as well as inhibition of lipoprotein lipase. This, together with stimulation of leptin, plasminogen activator inhibitor 1 and transforming growth factor β, inhibition of insulin receptor and IRS-1 autophosphorylation as well as reduction in GLUT-1 in

muscle cells, provokes insulin resistance in NIDDM and obesity. Interestingly, insulin resistance correlates with plasma sTNF-R2, but not sTNF-R1. Furthermore, TNF-R2 mRNA, but not TNF-R1 mRNA, is elevated in adipose tissue, as is sTNF-R2, but not sTNF-R1, in the circulation, and sTNF-R2 correlates with both BMI, hyperinsulinaemia and insulin resistance. Although we saw a correlation of sTNF-R2 with BMI in our hypertensive subjects, this was much weaker ($P = 0.07$) than that with blood pressure. An overall scheme, based on the present results and existing information, is shown in Figure 7 to illustrate a hypothesis that is consistent with the investigators' data. In this Figure, the new genetic findings from the present work are presented in the context of existing knowledge.

In the CAD population we found an association between the *CA16* allele and higher apo A-I. In this population, however, we do not find any association between the polymorphism and the severity of CAD, nor with the angiographically documented CAD. The association with a higher risk of CAD is a cross-sectional comparison between two populations. We did not measure apo A-I in the healthy control population. In the CAD patients, apo A-I levels are lower than for a healthy population. Apo A-I is cardioprotective. Apo A-I has an important role in the inflammatory response and may participate in inhibition of atherosclerosis. Apo A-I, but not HDL, has been found to inhibit IgG-induced neutrophil activation by ~60%, as measured by degranulation and superoxide production, and analogs having tandem repeating amphipathic helical domains are 10 times more effective in this regard than peptide analogs. Since the *CA16* allele is associated with CAD, the higher apo A-I in this genotype could reflect a protective response. The pathway that might lead from *TNFRSF1B* genotype to CAD is uncertain and may involve other factors (known, but not measured or not known, so not measured) that produce pathological changes that contribute to the abnormalities seen in CAD. The association with CAD was very strong. This was not just because of the high "n" value for this patient group, but a greater differential in the allele frequencies of the proposed disease-associated allele (*CA16*) in the patient group vs control, viz. 0.32 (CAD) cf. 0.26 (hypertensive) cf. 0.20 (control).

In NIDDM an association of the *CA16* allele was seen with neuropathy, but not with other complications. The pathogenic mechanism of neuropathy differs from that involved in vascular disease, so a finding for one complication, but not others, is possible. Recent evidence points to a role for an inflammatory response in neuropathy

and that immunosuppressive therapy may be beneficial to NIDDM neuropathy patients. The low glycosylated Hb in *CA16* homozygotes indicates improved glycemic control in this genotype. This would be consistent with a favourable effect on neurones. However, prevention of the development of peripheral neuropathy by free radical scavengers and inhibitors of TNF-a production such as N-acetylcysteine. pentoxifylline and gliclazide occurs irrespective of blood glucose levels. The determination of *TNFRSF1B* genotype may help predict the likelihood of developing diabetic neuropathy. NIDDM patients with the CA16 genotype may be deemed more at risk and treated with drugs such as gliclazide which have a beneficial effect on peripheral neuropathy. Allele *CA16* was associated with elevation in HDL-C. High HDL-C is associated with reduced vascular disease, which could explain in part the lack of association of *TNFRSF1B* genotype with vascular complications.

TNF-R2 also binds NGF, whose locus and variation in mRNA has been implicated in rat genetic hypertension, and could synergize with a *TNFRSF1B* genetic effect in hypertension etiology.

Other genetic factors would of course be needed to fulfil the summation of the genetic contribution to the totality of the blood pressure rise and hyperlipidemia in the hypertensive patients. Similarly, other genetic factors and effects contribute to CAD and NIDDM.

In conclusion, the present inventor has found a hypertension locus at 1p36.2, where *TNFRSF1B* resides. By themselves, the linkage data does not imply that *TNFRSF1B* is a causative gene in hypertension. However, the positive findings from a subsequent association study, which confines the focus to a much narrower region of the genome, suggests that *TNFRSF1B* may indeed be a gene responsible for hypertension. Coupled with further data, the inventor argues that a *TNFRSF1B* genotype that is more responsive to TNF could be involved in the causation of essential hypertension. The finding of an influence of *TNFRSF1B* genotype on plasma cholesterol in the hypertensive patients studied suggests a possible generalized role in insulin resistance. This may explain the association also observed with NIDDM. The strongest association was, however, with CAD, suggesting an important role in this life-threatening condition. The causative variant remains to be identified, but may reside in promoter DNA or involve an amino acid difference that affects receptor shedding or affect the properties of the receptor protein in other ways, such as ligand binding affinity. Moreover, pathophysiological mechanisms leading to disease phenotype in each case

require further research in order for the overall disease process related to *TNFRSF1B* to be unravelled.

The *TNFRSF1B* marker, together with other markers near this gene and other genes in the vicinity (e.g., *D1S2834*) and other loci should assist in (1) prediction of susceptibility to CAD, hypertension, NIDDM and associated pathophysiological changes early in life, and (2) diagnosis of the underlying cause in individuals with one or more of these conditions, which are well recognized as being heterogeneous. In addition, the findings suggest that new therapies directed specifically at TNF-R2 or its gene or other mechanisms that regulate TNF-R2 production could find utility in vasoprotective, antihypertensive, lipid-lowering or diabetic neuropathy therapy.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. A method of determining a predisposition in a subject to a complex polygenic disease, the method including assaying chromosome 1 for a genetic marker indicative of a predisposition to the complex polygenic disease.

2. A method of claim 1 wherein the complex polygenic disease is selected from the group consisting of coronary artery disease, essential hypertension, hyperlipidemia, non-insulin-dependent diabetes mellitus and neuropathy in non-insulin-dependent diabetes mellitus.

3. A method according to claim 1 or claim 2 wherein the genetic marker is a polymorphism.

4. A method according to any one of the preceding claims wherein the genetic marker is in region 1p36.2 of chromosome 1.

5. A method according to any one of the preceding claims wherein the genetic marker is a polymorphism in or near the TNF-R2 gene (*TNFRSF1B*).

6. A method according to claim 5 wherein the polymorphism is a dinucleotide repeat.

7. A method according to claim 6 wherein the dinucleotide is CA.

8. A method according to claim 6 or claim 7 wherein the polymorphism is a multi-allelic microsatellite polymorphism located in intron 4 of *TNFRSF1B* or is a multi-allelic microsatellite polymorphism *D1S2834*.

9. A method according to claim 8 wherein the allele(s) of the polymorphism are within a dinucleotide repeat running within intron 4 of *TNFRSF1B* from 33 to 67 base pairs 3' of the most 3' nucleotide of exon 4, and wherein a variable number of dinucleotide repeats giving a difference in length of the overall block of repeating units that are most commonly of up to 10 base pairs.

10. A method according to claim 9 wherein the alleles of a polymorphism are selected from at least one of the group consisting of the *CA13* allele, the *CA14* allele, the *CA15* allele, the *CA16* allele, the *CA17* allele, the *CA10* allele, the *CA11* allele, the *CA12* allele, the *CA18* allele and the *CA19* allele, the latter five (namely *CA10*, *CA11*, *CA12*, *CA18*, and *CA19*, being rare).

11. A method according to claim 10 wherein the regions of interest are selected from at least one of the group consisting of 267 bp (*CA13* allele), 269 bp, (*CA14* allele), 271 bp (*CA15* allele), 273 bp (*CA16* allele) and 275 bp

(*CA17* allele), and rarely 263 bp (*CA11* allele), 265 bp (*CA12* allele), 277 bp (*CA18* allele), 279 bp (*CA19* allele), embracing within the dinucleotide repeat polymorphism the first repeat of which begins approximately 33 bp 3' of the end of exon 4 of *TNFRSF1B* and the first of the more common alleles of which (allele *CA13*) begins after the 57th nucleotide 3' of exon 4.

12. A method according to any one of the preceding claims wherein the assay is carried out using DNA amplification, sequencing, allele-specific hybridization. DNA hybridization or ligase technologies.

13. A method according to claim 12 wherein the amplification is performed by the polymerase chain reaction (PCR).

14. A method according to claim 13 wherein PCR is used to amplify the intron 4 region of *TNFRSF1B* in which the polymorphism of interest is likely to exist from extracted DNA from a biological sample obtained from the individual.

15. A method according to claim 14 wherein the PCR primers are SEQ ID NO: 2 and SEQ ID NO: 3.

16. A method according to claim 14 wherein the PCR primers are SEQ. ID. NO: 3 and SEQ. ID. NO: 4.

17. A method of complex polygenic disease therapy including:

screening a subject for a predisposition to a complex polygenic disease correlating with a genetic marker on chromosome 1; and

if such predisposition is identified. treating the subject to prevent or reduce the complex polygenic disease or to delay its onset.

18. A method according to claim 17 wherein the complex polygenic disease is selected from the group consisting of coronary artery disease, essential hypertension, hyperlipidemia, non-insulin-dependent diabetes mellitus and neuropathy in non-insulin-dependent diabetes mellitus.

19. A method according to claim 17 or claim 18 wherein the genetic marker is in region 1p36.2 of chromosome 1. A method according to any one of the preceding claims wherein the genetic marker is a polymorphism in or near the TNF-R2 gene.

20. A method according to claim 19 wherein the polymorphism is a dinucleotide repeat.

21. A method according to claim 20 wherein the dinucleotide is CA.

22. A method according to claim 20 or claim 21 wherein the polymorphism is a multi-allelic microsatellite polymorphism located in intron 4 of *TNFRSF1B* or is a multi-allelic microsatellite polymorphism *D1S2834*.

23. A method for diagnosing a genetic predisposition in a subject for a complex polygenic disease(s), including

obtaining a biological sample from the subject; and

assaying chromosome 1 for a genetic marker indicative of a predisposition to the complex polygenic disease.

24. A method of claim 23 wherein the complex polygenic disease is selected from the group consisting of coronary artery disease, hypertension, hyperlipidemia and non-insulin-dependent diabetes mellitus.

25. A method according to claim 23 or claim 24 wherein the genetic marker is a polymorphism.

26. A method according to any one of claims 23 to 25 wherein the genetic marker is in region 1p36.2 of chromosome 1.

27. A method according to any one of the preceding claims wherein the genetic marker is a polymorphism in or near the TNF-R2 gene.

28. A method according to claim 27 wherein the polymorphism is a multi-allelic microsatellite polymorphism located in intron 4 of *TNFRSF1B*.

29. A method according to any one of claims 23 to 28 wherein the assay is carried out using PCR.

30. A PCR primer selected from the group consisting of SEQ. ID. NO: 2, SEQ. ID. NO: 3, SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 8 and SEQ. ID. NO: 9

31. A diagnostic kit including a pair of primers selected from SEQ. ID. NO: 2 and SEQ. ID. NO: 3 or SEQ. ID. NO: 4 and SEQ. ID. NO: 3 or SEQ. ID. NO: 8 and SEQ. ID. NO: 9.

32. In a method of testing an individual with a family history or in the early stages of complex polygenic disease to ascertain the chance of developing hypertension, neuropathy, and/or lipid disturbances including high total cholesterol, high low density lipoprotein cholesterol, abnormal apolipoprotein A1, abnormal glycosylated hemoglobin, the method including assaying chromosome 1 for a genetic marker indicative of a predisposition to the cardiovascular disease.

33. A method of claim 32 wherein the complex polygenic disease is selected from the group consisting of coronary artery disease, hypertension, hyperlipidemia and non-insulin-dependent diabetes mellitus.

34. A method according to claim 32 or claim 33 wherein the genetic marker is a polymorphism.

35. A method according to any one of claims 32 to 34 wherein the genetic marker is in region 1p36.2 of chromosome 1.

36. A method according to any one of claims 32 to 34 wherein the genetic marker is a polymorphism in or near the TNF-R2 gene (*TNFRSF1B*).

37. A method according to one of the claims 33 to 37 wherein the polymorphism is a multi-allelic microsatellite polymorphism located in intron 4 of the TNF-R2 gene (*TNFRSF1B*).

38. A method according to one of the claims 33 to 36 wherein the polymorphism is a multi-allelic polymorphism *D1S2834*.

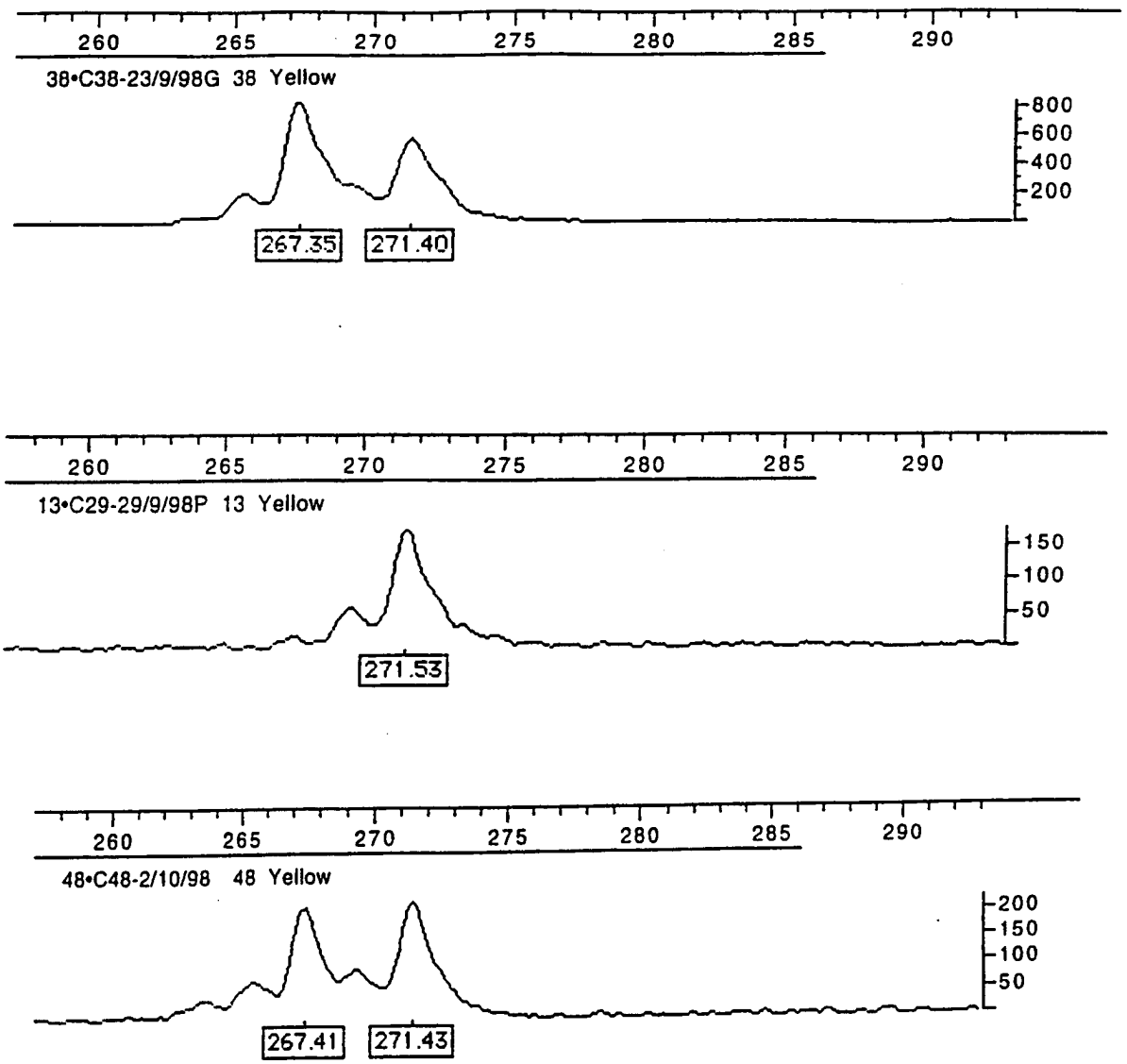
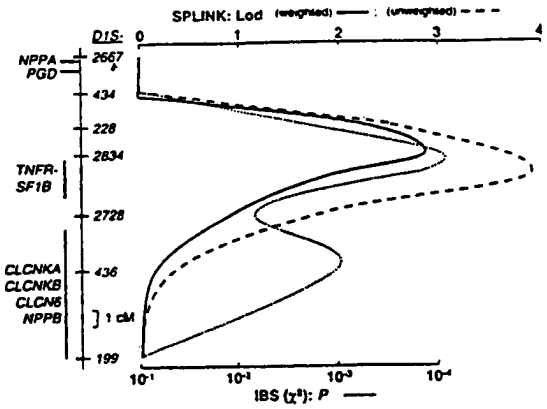
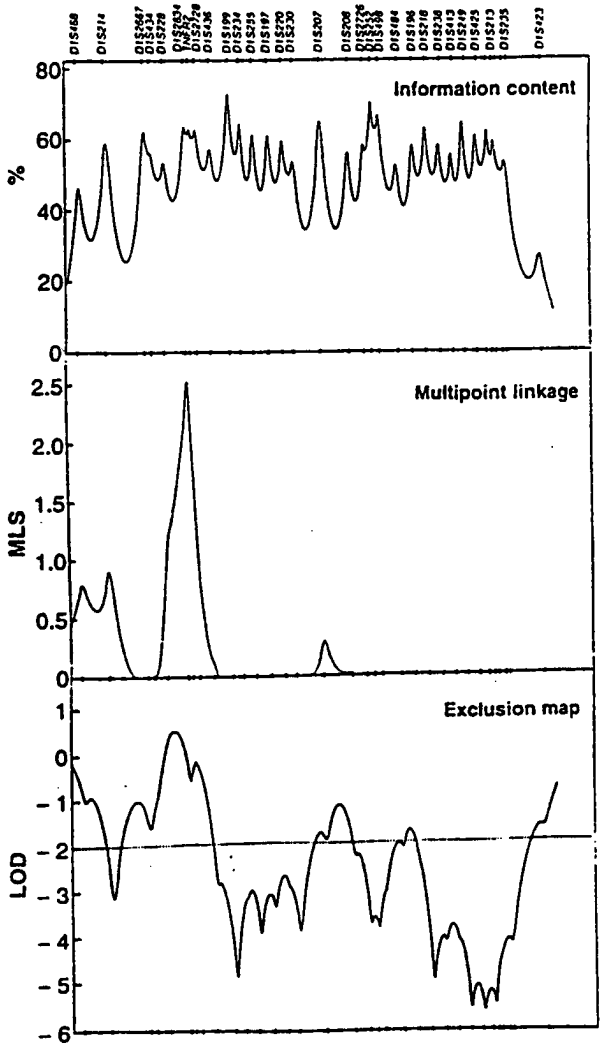

260
265
270
275
280
285
290
38•C38-23/9/98G 38 Yellow
800
600
400
200
267.35
271.40
260
265
270
275
280
285
290
13•C29-29/9/98P 13 Yellow
150
100
50
271.53
260
265
270
275
280
285
290
48•C48-2/10/98 48 Yellow
200
150
100
50
267.41
271.43

FIGURE 1



(a)

ESSENTIAL HYPERTENSION LOCUS ON CHROMOSOME 1



(b)

FIGURE 2

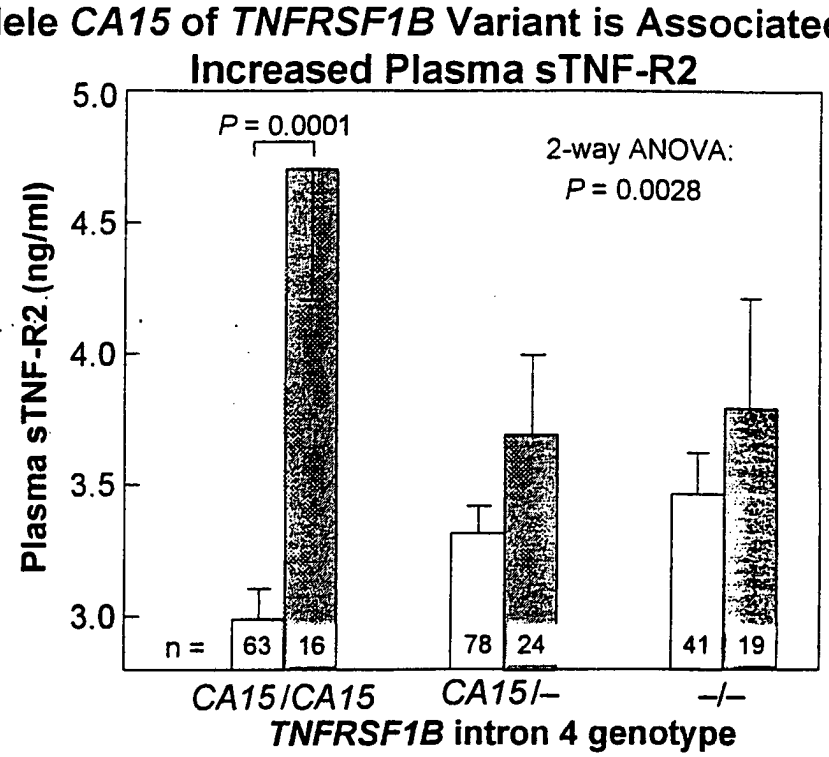

Allele CA15 of TNFRSF1B Variant is Associated with Increased Plasma sTNF-R2
Plasma sTNF-R2 (ng/ml)
5.0
4.5
4.0
3.5
3.0
P = 0.0001
2-way ANOVA:
P = 0.0028
n =
63
16
78
24
41
19
CA15/CA15
CA15/–
–/–
TNFRSF1B intron 4 genotype

Fig. 3

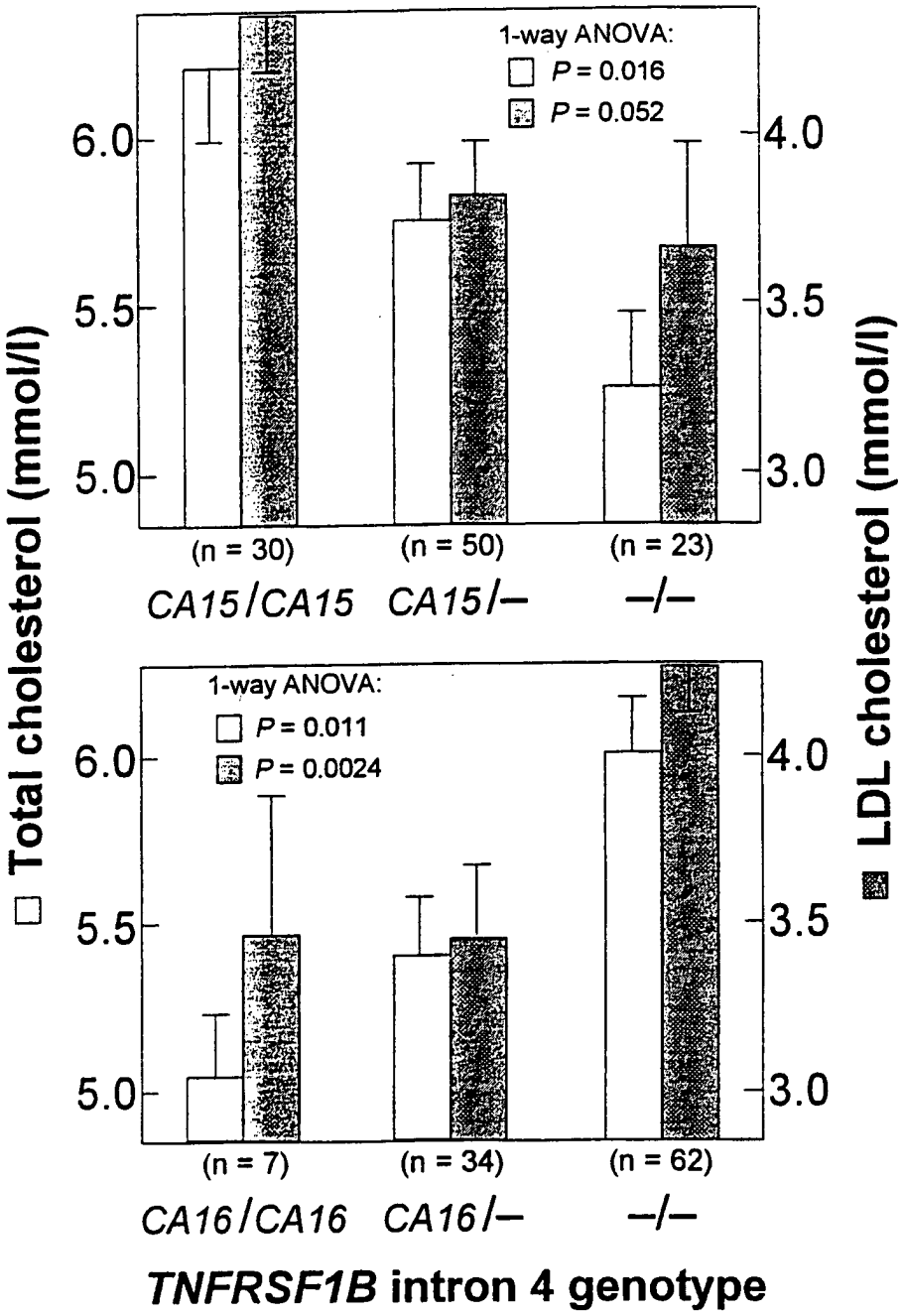


Fig. 4

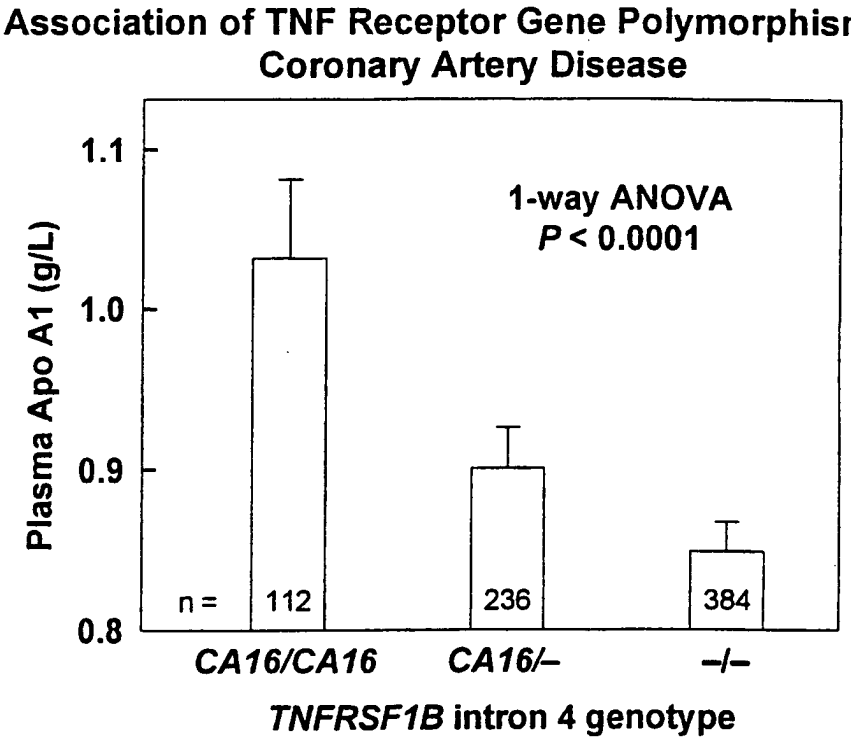

Association of TNF Receptor Gene Polymorphism with Coronary Artery Disease
Plasma Apo A1 (g/L)
1.1
1.0
0.9
0.8
1-way ANOVA
P < 0.0001
n =
112
236
384
CA16/CA16
CA16/–
–/–
TNFRSF1B intron 4 genotype

Fig. 5

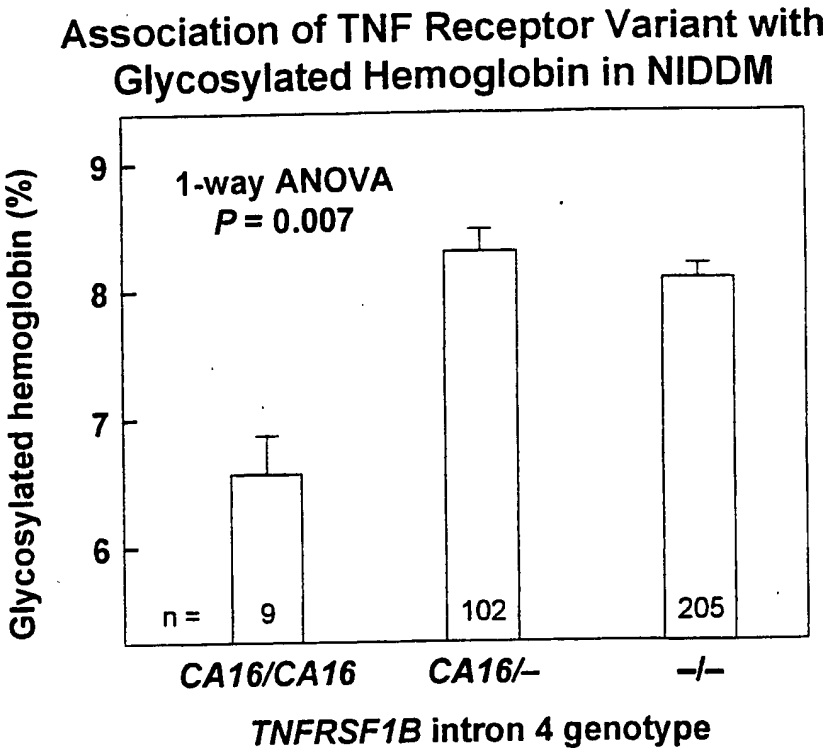


Fig. 6

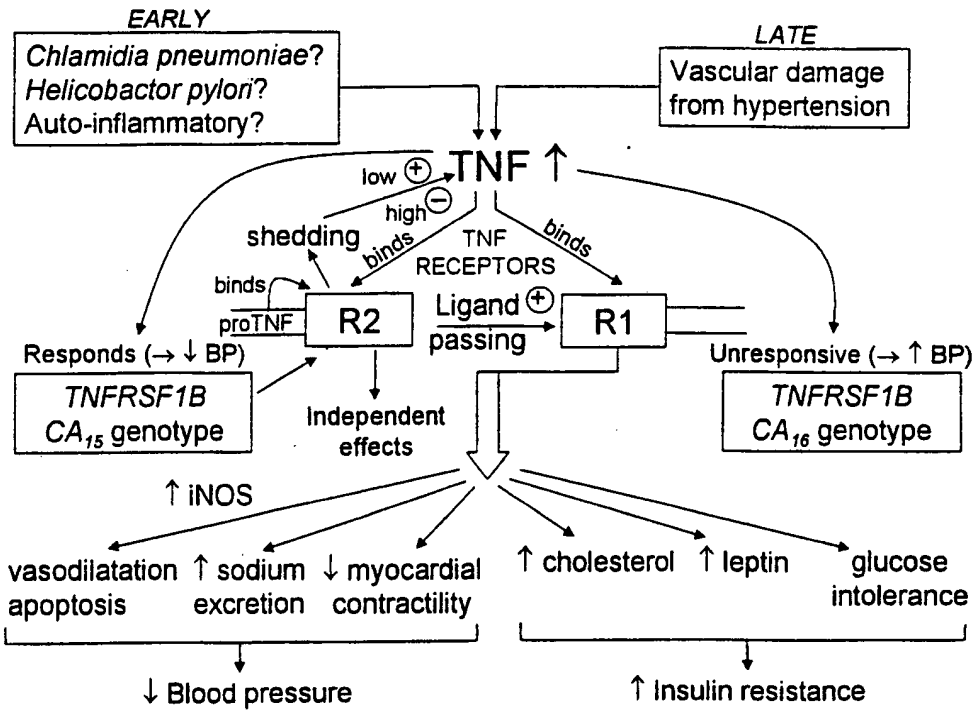

EARLY
Chlamidia pneumoniae?
Helicobactor pylori?
Auto-inflammatory?
LATE
Vascular damage from hypertension
TNF ↑
low ⊕
high ⊖
shedding
binds
TNF RECEPTORS
binds
binds
proTNF
R2
Ligand ⊕ passing
R1
Responds (→ ↓ BP)
TNFRSF1B CA15 genotype
Independent effects
Unresponsive (→ ↑ BP)
TNFRSF1B CA16 genotype
↑ iNOS
vasodilatation
apoptosis
↑ sodium excretion
↓ myocardial contractility
↑ cholesterol
↑ leptin
glucose intolerance
↓ Blood pressure
↑ Insulin resistance

Fig. 7

SEQUENCE LISTINGS

(1) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 170 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

Shown is CA16-repeat allele. The intron 4 repeated sequence begins 33 bp 3' of exon 4. Note that the term "CA repeat" is the same as "GT" repeat since one will be the sequence read from one strand of DNA and the other will be read from the complementary strand of DNA where CA (GT) repeats occur.

AATCTGTGTG TGTGCATGTG TGTACAGCAT CTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTAAGGGG TGGAGGTGCA GACAGAGCTC CTTGGGCCCC TCAGACCTCT CCTAGGGCTC TAGTGCCAAG GCCCAGCTGT CCCGCAGAGT GTCTGAGTGG TTGACCAAG

(2) INFORMATION FOR SEQ ID NO: 2

(ii) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

(forward primer for detection of *TNFRSF1B* intron 4 polymorphism)

GTGATCTGCA AGATGAACTC AC

(3) INFORMATION FOR SEQ ID NO: 3

(iii) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

(reverse primer for detection of *TNFRSF1B* intron 4 polymorphism)

ACACCACGTC TGATGTTTCA

(4) INFORMATION FOR SEQ ID NO: 4

(iv) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

(Forward primer for detection of microsatellite marker *TNFRSF1B* intron 4 polymorphic region)

AATCTGTGTG TGTGCATGTG

(5) INFORMATION FOR SEQ ID NO: 5

(v) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 bases

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5
(Reverse primer for detection of microsatellite marker *TNFRSF1B* intron 4 polymorphic region)

CTTGGTCAAC CACTCAGAC

(6) INFORMATION FOR SEQUENCE ID NO: 6
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6
(Forward primer for detection of microsatellite marker *D1S228*)

AACTGCAACA TTGAAATGGC

(7) INFORMATION FOR SEQUENCE ID NO: 7
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

(reverse primer for detection of microsatellite marker *D1S228*)

AACTGCAACA TTGAAATGGC

(8) INFORMATION FOR SEQ ID NO: 8

(viii) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

(forward forward primer for detection of microsatellite marker *D1S2834*)

TGTCGGATGT GGGCAG

(9) INFORMATION FOR SEQ ID NO: 9

(ix) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

(Reverse primer for detection of microsatellite marker *D1S2834)*

TATGAAATGG GGATAATAGT ACGG

(10) INFORMATION FOR SEQ ID NO: 10

(x) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10
(Forward primer for detection of microsatellite marker *D1S2728)*

CGCCTCGTTC AGTTCATA

(11) INFORMATION FOR SEQ ID NO: 11
(xi) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11
(Reverse primer for detection of microsatellite marker *D1S2728)*
ACTACACTCC AGCCTGGGT

(12) INFORMATION FOR SEQ ID NO: 12
(xii) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10
(Forward primer for detection of microsatellite marker *D1S436)*

TGAATGTGTC TCCAGTGTTA GC

(13) INFORMATION FOR SEQ ID NO: 13

(xiii) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

(Reverse primer for detection of microsatellite marker *D1S436)*

CTGTAGAGCA ATCTGGCAAT ATGT

(14) INFORMATION FOR SEQ ID NO: 14

(xiv) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

(Forward primer for detection of *TNFRSF1B* exon 6 polymorphism)

CCGTGAATGA GCCCAG

(15) INFORMATION FOR SEQ ID NO: 15

(xv) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15
(Reverse primer for detection of *TNFRSF1B* exon 6 polymorphism)

CAGAAGGAGT GAATGAATGA G

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01050

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12Q I/68; A61K

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
AS ABOVE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AS BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, Medline, WPAT: Keywords: Chromosome 1, polymorphism, diabetes, (cardiac/cardiovascular disease), hypertension, hyperlipidemia, (tumor necrosis factor receptor/TNF receptor/TNFR).

GenBank, EMBL, PDB Nucleic Acids: SEQ ID NO 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Hanson et al. An Autosomal Genomic Scan for Loci Linked to Type II Diabetes Mellitus and Body-Mass Index in Pima Indians. American J Hum Genet. 1998. **63**: 1130-1138	1-3,12, 13,17, 18, 23-25, 32-34.
X	Mansfield et al. Multilocus linkage of familial hyperkalaemia and hypertension, pseudohypoaldosteronism type II, to chromosomes 1q31 and 17p11-q21. Nature Genetics. 1997. **16**: 202-205.	1-3,12, 13,17, 18, 23-25, 32-34.

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1999	06 JANUARY 2000 (06.01.00)
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **GILLIAN ALLEN** Telephone No.: (02) 6283 2266

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01050

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Griffiths et al. A Locus on the Long Arm of Chromosome 1 as a Possible Cause of Essential Hypertension. 1991. **18**: 363-366.	1-3,12, 13,17, 18, 23-25, 32-34.
X	Pajukanta et al. Linkage of familial combined hyperlipidaemia to chromosome 1q21-q23. Nature Genetics. **18**: 369-373.	1-3,12, 13,17, 18, 23-25, 32-34.
X	Castellani et al. Mapping a gene for combined hyperlipidaemia in a mutant mouse strain. Nature Genetics. 1998. **18**: 374-377. Particularly p 377 column 1	1-3,12, 13,17, 18, 23-25, 32-34.
Y	Beltinger et al. Physical Mapping and Genomic Structure of the Human TNFR2 Gene. 1996. Genomics. **35**: 94-100.	1-5, 12, 13, 17-19, 23-27, 32-36
Y	Testa et al. Circulating Levels of Cytokines and their Endogenous Modulators in Patients with Mild to Severe Congestive Heart Failure due to Coronary Artery Disease or Hypertension. 1996. JACC. **28**(4): 964-971.	1-5, 12, 13, 17-19, 23-27, 32-36
Y	Hofmann et al. Altered Gene Expression for Tumor Necrosis Factor-□ and its Receptors during Drug and Dietary Modulation of Insulin Resistance. Endocrinology. 1994. **134**(1): 264-270.	1-5, 12, 13, 17-19, 23-27, 32-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01050

Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos..
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 12, 13, 17, 23, 25, 29, 32 and 34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The term "complex polygenic disease" is not defined in terms of particular diseases. The term is considered indeterminate and it is not considered possible to meaningfully search claims containing this term.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The claims are unified by the method step of assaying chromosome 1 for genetic markers indicative of particular disease states. However, genetic markers on chromosome 1 associated with the diseases of claim 2 are already known. Therefore the unifying factor is not novel, and there is *a postiori* lack of unity between the different genetic markers of the claims, and there are multiple inventions, one invention for each polymorphic locus claimed.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.